

Complex Trait of Acute Pancreatitis:

Studies of candidate genes and adipokines

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Academic Dissertation

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ABSTRACT

Acute pancreatitis (AP) is a common disease for which are attributable over 4000 hospitalization periods per year in Finland. Clinical symptoms include abdominal pain, nausea, vomiting, tachycardia, and takypnoe. The disease is most often associated with alcohol consumption, though biliary disease-induced AP is also common. Mild disease resolves spontaneously in a few days. Severe forms of the disease can lead to local complications, necrosis, and abscesses in and around the pancreas. Systemic inflammation in severe AP is associated with distant organ failures. Respiratory failure is the most common form of organ failure. Multiple organ failures carry high risk for lethal outcome. The severity of the developing AP has been an enigma for physicians for decades and has produced a considerable amount of scientific research. The factors that have been identified as associating with severe AP are age, co-morbid conditions and obesity.

The aim of this study is to identify genetically determined prognostic factors involved in the clinical features of AP. The study employs a candidate-gene approach, and the genes are involved in trypsinogen activation in the initiation phase of the disease, as well as in the systemic inflammation as the disease proceeds. The last study examines adipokines, fat-derived hormones characterized with the capacity to modify inflammation.

SPINK 1 is a gene coding trypsin activation inhibitor. Mutations in sites N34S and P55N occur in patients with idiopathic and hereditary pancreatitis. These mutations were determined by minisequencing methods in 371 AP patients and in 459 controls. The mutation N34S was more common in AP patients (7.8%) than in controls (2.6%). This suggests that *SPINK 1* gene mutation N34S is a risk factor for AP.

The inflammatory gene polymorphisms of *TNF*, *HSPA1B*, *CD14* and *IL-10* have been suggested to be involved in risk for alcoholic AP or in AP disease severity. In the study material of 397 AP patients and 310 controls the genotypes were determined by MALDI-TOF-assisted methods. No differences could be identified between the different study groups.

Hemostatic changes are closely associated with inflammation, suggesting that genetic changes favoring coagulation might be those factors associated with a more severe inflammatory response. Prothrombotic polymorphisms of coagulation Factor V gene (Leiden mutation) and plasminogen activator inhibitor-1 (4G/5G promoter polymorphism) gene were determined in 397 AP patients. In our study population, the allele frequency for the Factor V Leiden mutation and the plasminogen activator inhibitor-1 4G allele was comparable to that of the general population.

In the fourth study, in 12 matched pairs of patients with severe and mild AP, levels of adipokines, adiponectin, and leptin were evaluated on admission and during the first week of hospitalization. During the course of AP, plasma levels of adiponectin were constant. Plasma adipokine levels did not differ between patients with mild and severe AP. In patients with mild AP leptin plasma levels decreased. These results suggest that in AP, adipokine plasma levels are not factors predisposing to organ failures.

This study identified the *SPINK 1* mutation N34S to be a risk factor for AP in the general population. The inflammatory gene polymorphisms studied seem to play no role in alcohol-induced AP or in determining AP severity. The hemostatic polymorphisms studied showed no association with severe AP. Adipokines, adiponectin, and leptin seem to have no influence on systemic inflammation in clinical AP. As AP is a multifactorial disease, and extensive genetic heterogeneity is likely, further identification of genetic factors in the disease requires larger future studies with more advanced genetic study models. Further identification of the patient characteristics associated with organ failures offers another direction of the study to achieve more detailed understanding of the severe form of AP.

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LIST OF ORIGINAL PUBLICATIONS

The following original publications are referred to in the text by their Roman numerals I to IV.

- I Tukiainen E, Kylänpää M-L, Kemppainen E, Nevanlinna H, Paju A, Repo H, Stenman U-H, Puolakkainen P. Pancreatic secretory trypsin inhibitor (SPINK1) gene mutations in patients with acute pancreatitis. *Pancreas* 2005;30:239-42
- II Tukiainen E, Kylänpää M-L, Puolakkainen P, Kemppainen E, Halonen K, Orpana A, Methuen T, Salaspuro M, Repo H. Polymorphisms of the TNF, CD14 and HSPA1B genes in patients with acute alcohol-induced pancreatitis. *Pancreas*; *in press*.
- III Tukiainen E, Kylänpää M-L, Repo H, Kemppainen E, Orpana A, Methuen T, Salaspuro M, Puolakkainen P. Hemostatic gene polymorphisms in severe acute pancreatitis. *Submitted*.
- IV Tukiainen E, Kylänpää M-L, Ebeling P, Kemppainen E, Puolakkainen P, Repo H. Leptin and adiponectin levels in acute pancreatitis. *Pancreas* 2006;32:211-214

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ABBREVIATIONS

Gene symbols have been *ITALICIZED* in the text.

All gene symbols can be found at <http://www.ncbi.nlm.nih.gov/entrez>.

AP	Acute pancreatitis
APACHE II	Acute physiology and chronic health evaluation II
BMI	Body mass index
CRP	C-reactive protein
CT	Computed tomography
CD14	Cluster of differentiation membrane-associated glycosylphosphatidylinositol-linked protein 14
CP	Chronic pancreatitis
ELISA	Enzyme-linked immunosorbent assay
HLA	Human leukocyte antigen
HSP	Heat shock protein
HWE	Hardy-Weinberg equilibrium
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-10	Interleukin-10
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer
MHC	Major histocompatibility complex
OF	Organ failure
PAI-1	Plasminogen activation inhibitor 1
PCR	Polymerase chain reaction
RA	Receptor antagonist
SIRS	Systemic inflammatory response syndrome
SNP	Single nucleotide polymorphism
SPINK1	Serine protease inhibitor, Kazal type I
TNF α	Tumor necrosis factor α

1 INTRODUCTION

Patients with acute pancreatitis (AP) present with abdominal pain, nausea, and vomiting. Upper abdominal tenderness is frequently detected in clinical examination, and the pain often radiates to the back. In mild AP, uneventful recovery usually ensues in a few days following hospital admission. The clinical presentation of severe AP is severe abdominal pain, takypnea, tachycardia, oliguria, and shock, signs of Cullen and Gray Turner,¹ and death.

The most common etiologic factors for AP are alcohol or biliary stones. Incidence of AP in Finland is 80/100 000 per year, and familial pancreatitis is rare. No specific treatment exists. Treatment is symptomatic and includes pain relief and intravenous crystalloids in mild cases. As the disease turns into a severe form, patients receive prophylactic antibiotics. In severe AP, in addition to local complications in the pancreas, distant organ failures (OF) supervene. These patients receive treatment in intensive care units and need assistance regarding many organ functions.

Genetic research in the field of AP has received no focus before this century. When studying complex genetic traits, precise definitions of phenotypes (trait components) are the starting points of the study. In a population of patients with AP, homogenous subgroups are identified based on clinical criteria. Differing etiologic factors and degree of disease severity, as well as complications of the disease categorize the patients.

The severity of developing disease has been an enigma for physicians for decades and has produced a considerable amount of scientific research. The pathognomic processes involved in the progression of the disease are widely characterized. These involve inappropriate trypsin activation in the pancreas and activation of cellular and humoral inflammatory cascades. Systemic inflammation in AP includes production of cytokines, as well as disturbances in the coagulation system. The growing knowledge and detailed functional characterization of single nucleotide polymorphism has promoted genetic study of all the complex traits. Genotyping techniques are developing, and feasible genotyping techniques are now available.

Finns have a common genetic background, and in that sense Finland offers an attractive field for a genetic study. Alcohol-induced AP is extraordinarily common in Finland compared to rates in other countries, meaning that some special genetic features may be responsible for the high Finnish incidence.

This study applies the candidate-gene approach in series of 700 AP patients and controls. The aim is to identify genetic features that are prognostic factors associated with clinical aspects of AP.

2 REVIEW OF THE LITERATURE

2.1 Clinical Aspects of Pancreatitis

Pancreatitis is an inflammation of the pancreas, and the acute variety occurs suddenly, lasts for a short period of time, and usually resolves. Repeated episodes of AP may lead to chronic pancreatitis (CP), but the chronic form may also be triggered by only one acute attack, especially if the pancreatic ducts are damaged. CP results in a slow destruction of the pancreas. In CP, the patient has symptoms of pancreatic exocrine and endocrine dysfunction and continuous pain, and occasionally bursts of pancreatic inflammation resembling the clinical picture of AP.

Epidemiology of acute pancreatitis

The incidence of AP has risen during the past few decades in many countries.^{2,3,4} This rise is associated with increased alcohol consumption and possibly with a rising prevalence of obesity and accompanying gallstone disease.^{5, 6, 7} The incidence in Finland is extremely high and was estimated to be 73 cases/100 000 inhabitants per year.⁸ In contrast, in Norway the incidence is 30 cases/100 000.⁹ In Sweden, a novel decline in alcohol-induced AP has been detectable; however, at the same time, biliary AP has become more common, resulting in a rise in total AP incidence.¹⁰

A factor influencing AP incidence is ethnicity. The hospitalization rate for AP in the United States is higher in blacks than in whites.^{4, 11} The alcoholic etiology of AP is extremely common in Finland, up to 80% of cases.¹²

Table 1. Incidences of acute pancreatitis (AP) with proportions of alcohol-induced AP.

Country, year ^{reference}	Incidence of AP (cases /100 000)	Proportion of alcohol-induced AP (%)
Germany 1995 ¹³	20	32
Norway 1995 ⁹	20	19
Ireland 2004 ¹⁴	23	23
Iceland 1999 ¹⁵	32	32
Sweden 1999 ¹⁶	35	25
USA, California 2001 ¹¹	44	20
Finland 1989 ⁷	73	60

Mild and severe acute pancreatitis

Clinical diagnosis of AP is based on clinical evaluation and detection of elevated amylase or lipase activity in serum, plasma or urine, but normal amylase activity does not rule out an AP diagnosis.¹⁷ In cases with normal amylase levels, computed tomography (CT) can verify the diagnosis.¹⁸ Differential diagnosis of AP includes peptic ulcer disease, abdominal perforations and peritonitis, appendicitis, acute myocardial infarction, and other conditions.

In the majority of patients, the course of AP is mild and self limiting; abdominal pain is relieved in a few days by general supportive care, and local and systemic complications are rare. Early prediction of the severity of an acute attack has important implications for management and intervention. Severe AP is characterized by local complications in the pancreas and distant OF compromising survival. The precise definition between mild and severe AP should be unambiguous and has been the topic of continuous debate for pancreatologists.

Ranson and colleagues provided the first prognostic criteria for AP in 1974.¹⁹ These criteria consist of eleven signs evaluated within 48 hours of admission. Identification of three or more positive signs is prognostic for severe AP. Sign numbers higher than six are associated with mortality of more than 50% and systemic

complications. The Ranson factors also correlate with pancreatic necrosis.²⁰ Later, Imrie in Glasgow modified the scoring system by simplifying it to eight points.²¹

The Atlanta severity classification was established in the International Symposium on AP in Atlanta, Georgia, in 1992. It is a clinically based classification system for AP.²² According to this classification, severe AP is associated with OF alone or with local complications such as necrosis, abscesses or pseudocysts. Furthermore, accumulation of three or more Ranson criteria or eight or more Acute Physiology and Chronic Health Evaluation II (APACHE II) points²³ characterizes severe AP. The Atlanta classification is retrospective in nature and was developed for comparison of patient series. While the Atlanta classification may be ambiguous, its revision has been proposed.^{24, 25}

APACHE II, Multiple Organ Dysfunction Score,²⁶ and Sequential Organ Failure Assessment²⁷ scores are examples of scoring in assessing OFs. OF in AP correlate closely with mortality,^{12, 28} and thus OF scores are informative also in profiling AP patients.

The gold standard for laboratory assessment of severity of AP is C-reactive protein (CRP) concentration.²⁹ A value for CRP concentration > 150 mg/l has a specificity of 90% for severe AP on admission to hospital.³⁰ Contrast-enhanced CT is also a widely used means to classify AP severity.^{31, 32}

Alcohol-induced acute pancreatitis

Only a minority of alcoholics develop pancreatitis, suggesting individual susceptibility to the disease.^{33, 34, 35, 36} This implies some individual risk factors or alternatively, protective factors. It has also been noted that the incidence of alcoholic pancreatitis is proportional to amount of alcohol consumption, suggesting the presence of dose-related effects of alcohol on the pancreas.^{37, 38}

It is estimated that about 10 to 20% of individuals who consume large amounts of alcohol eventually develop pancreatitis.³⁹ It probably makes no difference whether the ethanol is consumed as wine, beer, or spirits, but the daily consumption in patients with pancreatitis averages 100-150 g/day.⁴⁰ It has also been suggested that not the continuous drinking but the withdrawal period is crucial in triggering the first episode.⁴¹ Another postulate is that the amount of alcohol consumed during the week of the first attack of AP correlates with disease severity.⁴²

The mechanisms by which alcohol triggers AP are not solidly defined.⁴³ It is likely that multiple factors together predispose to the disease.⁴⁴ The effects of alcohol and its metabolites and its metabolic by-products (reactive oxygen species)⁴⁵ may lead both to increased content of digestive and of lysosomal enzymes, and also to increased potential for contact between digestive and lysosomal enzymes in the pancreatic acinar cell. One cause is increased organelle fragility mediated by compounds such as cholesteryl esters, fatty acid ethyl esters, and reactive oxygen species.⁴⁶ These changes may facilitate premature intracellular activation of digestive enzymes and may predispose the gland to autodigestive injury and necroinflammation, if an appropriate trigger factor is present.

Among such trigger factors is smoking. Cigarette smoking alone may induce pancreatitis, or it may have an additive effect with alcohol. Different dietary components may interact and modify the effects of alcohol on the pancreas. The poor vitamin status of patients with alcoholic AP may be contributory.⁴⁷ Ethnicity may be a contributory factor, for example afroamerican individuals may be at increased risk for alcohol-induced pancreatitis.^{48, 49} Furthermore, viral infections and bacterial endotoxin may also be triggering cofactors.^{50, 51, 52}

Treatment of acute pancreatitis

No specific treatment exists. The usual treatment is to give intravenous fluids to prevent dehydration, plus sufficient pain control. Treatment of AP is mainly symptomatic and supportive. During the first days the patients are not given any food until the symptoms disappear. However, in cases with severe AP, enteral feeding is recommended and started with a nasojejunal catheter during the first few days.⁵³ Patients with severe AP receive intravenous fluids, prophylactic and target-specific antibiotics, and supportive care in an intensive care unit. Abdominal compartment syndrome, gut necrosis, and uncontrollable hemorrhage are reasons for surgical intervention in the early phase of the severe disease.⁵⁴ After two weeks of severe AP, infected necrosis is the most common indication for surgery.^{55, 56}

Prognosis of acute pancreatitis

Mortality rates

In recent reports, hospital mortality from severe AP was between 15 to 30%.^{57, 58} The reduction in mortality from AP during the last two decades is attributed to early identification of severe AP and appropriate intensive care unit management.⁵⁹ Many AP patients die within the first two weeks of onset.^{60, 61} Another peak in mortality occurs in the late phase, weeks after the onset of the disease, and is associated with infectious complications of pancreatic necrosis.^{62, 63} Approximately one-third of all deaths due to AP are outpatient fatalities, and these deaths are strongly associated with social isolation and alcohol abuse.^{64, 65}

Risk factors

Systemic inflammatory syndrome (SIRS) that persists over 48 hours is associated in AP patients with multiple organ dysfunction and death.⁶⁶ Persistent SIRS has been associated with a mortality of 25% compared to lower than 10% in those SIRS resolving in less than 48 hours.⁶⁷ Advanced age, chronic comorbid conditions, and need for dialysis, mechanical ventilator support, and vasopressor support are prognostic factors for a fatal outcome.^{68, 69}

Obesity is a clear risk factor for AP. It is not only a risk factor for the development of local and systemic complications; it also leads to increased mortality.^{70, 71, 72}

Alcoholic pancreatitis is an etiologic factor associated with higher mortality rates⁷³ and with more frequent development of pancreatic necrosis^{74, 75} than in other forms of AP. This finding is not consistent, however.⁷⁶ Male gender has been suggested to be a risk factor for severe AP; this, however, remains controversial.^{77, 78}

Long-term prognosis

Detrimental effects on patient health due to AP include exocrine and endocrine pancreatic insufficiencies that correlate with amount of pancreatic necrosis.⁷⁹ Approximately half the patients develop impaired glucose intolerance after severe AP.⁸⁰ Insulin-dependent diabetes mellitus after severe AP is a more rare manifestation, presenting in approximately 10% of cases.⁸¹ Repeated episodes of alcoholic AP may lead to

CP. Up to 13% of severe AP patients surviving their initial hospitalization die within a few years. Among the survivors, long-term health-related quality of life is comparable to that of the normal population.⁸²

2.2 Pathogenesis of Acute Pancreatitis

The pancreas is located retroperitoneally in the upper abdomen, behind and below the stomach, and is connected to the intestinal tract by the pancreatic duct which opens into the duodenum. The pancreas can be divided into two separate units, one endocrine and one exocrine. The endocrine pancreas regulates energy metabolism by secretion of various hormones into the blood stream and is structurally located in distinct clusters of endocrine cells called the islets of Langerhans. The blood supply of the endocrine pancreas is provided by one to five arterioles per islet.⁸³ The islets of Langerhans comprise 1% of the pancreatic mass, but receive up to 15% of the organ's blood supply. The exocrine pancreas, the main part of the gland, secretes pancreatic juice into the intestinal tract. The pancreatic juice contains enzymes capable of digesting nutrients, and bicarbonate that neutralizes acidic gastric secretions.

Zymogen activation

Pancreatic digestive enzymes are stored as inactivated precursors in pancreatic zymogen granules. Under normal conditions, their activation is strictly controlled to prevent autodigestion of the pancreas; activation occurs in the small bowel. In certain circumstances, however, excessive amounts of pancreatic trypsinogen are activated to trypsin (ectopic activation), activating other downstream zymogens, and leading to autodigestion of the pancreas. Triggers for the activation of trypsinogen to trypsin in the pancreas include excessive pancreatic exocrine stimulation, reflux of bile or duodenal fluid, disturbance of pancreatic duct flow, and inflammation. Although enterokinase is the most efficient activator, other molecules activating trypsinogen include trypsin, lysosomal enzyme cathepsin B, and neutrophilic enzymes. Activation of trypsin from trypsinogen can also occur without enzyme involvement (non-enzymatic autoactivation). Calcium inhibits degradation (autolysis) of activated trypsin, whereas bile acids promote trypsin autoactivation.

SPINK1 (serine protease inhibitor, Kazal type 1), also called pancreatic secretory trypsin inhibitor or tumor-associated trypsin inhibitor, is synthesized in acinar cells of the pancreas and is thought to inhibit up to 20% of the pancreatic trypsin activity by binding to its catalytic site. Pancreatitis can develop if pancreatic activation of trypsinogen is too high, or the trypsin-binding ability of SPINK 1 is too low.^{84 85 86}

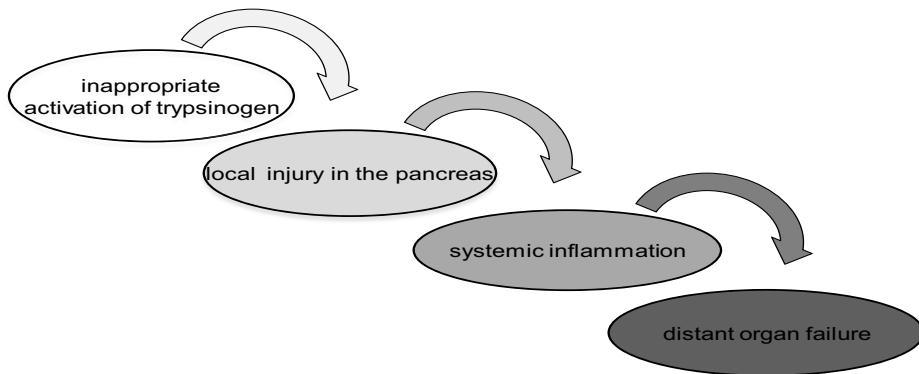


Figure 1. The cascade of pathogenic events in the progress of severe AP. Trypsinogen is the precursor of trypsin, the most important digestive enzyme produced in the pancreatic acinar cell. The most important inhibitor of pancreatic trypsin activity is SPINK1.

Autodigestion of the pancreas causes necrosis, apoptosis, and vascular damage in the pancreas, and various proteolytic and lipolytic enzymes are released and activated within the organ.⁸⁷ The noxious potential of elastase, lipase, chymotrypsin, and phospholipase A₂ exceeds that of trypsin in damaging acinar cells.

According to the “co-localization hypothesis,” during the early stages of AP, pancreas-derived digestive zymogens become co-localized with lysosomal hydrolases in acinar cell cytoplasmic vacuoles, and as a result of this co-localization, lysosomal hydrolases such as cathepsin B activate trypsinogen.⁸⁸ Furthermore, trypsin activates other digestive enzyme zymogens.

Neutrophils are recruited from the general circulation to the site of damage. Reactive oxygen species generated by infiltrating neutrophils are considered important regulators in the pancreatitis pathogenesis. The inflammatory cells, macrophages, fibroblasts, T-cells, and endothelial cells, along with damaged acinar cells, are responsible for the release of inflammatory mediators into the systemic circulation.

Systemic inflammation

Local injury in the pancreas may proceed to a systemic inflammatory response syndrome (SIRS). This syndrome is characterized by tachypnoea, tachycardia, high leukocyte count, and abnormal body temperature.⁸⁹ Persistent SIRS is associated in AP with multiple organ dysfunction syndrome and death and is an early indicator of AP severity.

The mediators of systemic inflammation in the pancreatitis include a variation in cells and cytokines. Nuclear factor κ B (NF- κ B) is a nuclear transcription factor responsible for regulating the transcription of a wide variety of genes involved in the inflammation in AP.⁹⁰ During severe AP, the pro- and anti-inflammatory cytokine response occurs early and persists in the systemic circulation for several days.⁹¹ Severe AP has many similarities to sepsis syndrome and septic shock. The hemodynamic features of cardiovascular instability, reduced ejection fraction, and decreased systemic vascular resistance in each of these conditions are indistinguishable. In addition, similarities in the cytokine and inflammatory mediator profiles are striking, suggesting that the hemodynamic abnormalities may result from the same pathogenic mechanisms.

In addition to hyper-cytokinaemia resulting from the inflammatory process in the pancreas and peripancreatic tissues, evidence exists that when AP is complicated by infection these cytokines are released by hyperactive macrophages.⁹² Cytokines activate neutrophils that have already infiltrated vital organs such as the lung, liver, and digestive organs. By excreting proteolytic enzymes, neutrophils injure the infiltrated vital organs, causing cellular damage and dysfunction of vital organs distant from the pancreas.

Although septic complications of severe AP do arise, these are usually late features. In the early phase of a severe attack, sterile pancreatic necrosis occurs. Evidence suggests that the important cytokines in the development of complications and multiple OF in severe AP are tumor necrosis factor α (TNF α), interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-8.^{93, 94} In addition, endotoxin and other important inflammatory mediators including platelet activating factor and phospholipase A2 are implicated in the development of complications in both severe AP and sepsis.⁹⁵ Furthermore, trypsin and activation of circulating trypsinogen in AP may contribute to development of distant organ injury.⁹⁶

Cytokines

Tumor necrosis factor α

TNF α is produced in AP by acinar cells from the pancreas and by activated peripheral blood monocytes. The systemic up-regulation of TNF α production in AP is triggered by early-signaling molecules released from the pancreas that gain access to the general circulation. In AP, TNF α overproduction is pivotal in the induction of inflammatory genes, cell death, endothelial upregulation, and in the recruitment and activation of immune cells.⁹⁷ TNF α , which is rapidly cleared from the bloodstream, has been considered as a novel pharmacologic target for treatment. Although promising results have emerged from the laboratory, their use in clinical practice seems problematic.^{98, 99}

Interleukin-1

IL-1 shares several inflammatory properties with TNF α . IL-1 is detectable within the pancreas early in the course of experimental AP.¹⁰⁰ Upregulation of IL-1 occurs not only within the pancreas, but during the course of AP also in distant organs. In addition to high levels of IL-1 in severe AP, levels of IL-1 receptor antagonist (RA) are also higher.¹⁰¹ Because negative feedback occurs between IL-1 and IL-1RA, IL-1 RA may attenuate AP severity.^{102, 103, 104}

Interleukin-6

IL-6 is important in the induction of synthesis of acute-phase proteins and is a clearly defined marker of disease severity in AP.^{105, 106} Experimental models have shown that IL-6 has implications in the development of the disease.¹⁰⁷

Interleukin-10

Early during the clinical AP, IL-10 plasma levels peak.¹⁰⁸ In clinical settings, IL-10 plasma levels are quite useful in predicting severe AP and OF on admission to hospital.^{109, 110, 111, 112, 113} This cytokine ameliorates AP in experimental settings.^{114, 115, 116} Due to its anti-inflammatory properties, IL-10 has been suggested for use as a drug to ameliorate AP; however, clinical trials in humans have been unsuccessful.¹¹⁷

Other factors

CD14 and endotoxin

CD14, a membrane-associated protein expressed on the surface of cells, especially of macrophages, acts as a receptor for detection of bacterial lipopolysaccharide, i.e., endotoxin. Intestinal permeability to various compounds and endotoxin is higher among patients with severe AP than among those with mild AP.¹¹⁸ The presence of endotoxemia predicts poor outcome in AP.¹¹⁹

Animal models have demonstrated that endotoxin potentiates lung injury,¹²⁰ and the anti-CD14 antibody ameliorates severe AP and pancreatic injury.¹²¹ CD14-deficient mice develop biochemical manifestations of AP, but their histological changes are more subtle than in controls.¹²² Endotoxin has been suggested to act as a cofactor during alcohol-induced AP in pancreatic necrosis of cells. The pancreas exposed to alcohol is more sensitive to endotoxin-induced damage, due to its increased sensitivity to necrotic rather than apoptotic cell death.¹²³

Heat shock proteins

Heat shock proteins (HSPs) are a group of proteins whose expression is increased when cells are exposed to elevated temperatures or other stress. HSPs, cytoprotective molecules that help to maintain the metabolic and structural integrity of cells, are designated according to molecular weight, for example, HSP70 refers to a family of heat shock proteins on the order of 70 kilodaltons in size. HSPs are upregulated in AP^{124, 125} and have protective effects in AP.^{126, 127, 128, 129, 130}

Hemostatic factors

Related to systemic inflammation in AP are abnormalities in coagulation and fibrinolysis.^{131, 132} Consumptive coagulopathy is demonstrated by decreased platelet counts, decreased prothrombin values, and consumption of fibrinogen during the first days of severe attacks.¹³³ Severe hemostatic disorders in AP may range from scattered intravascular thrombosis to disseminated intravascular coagulation.^{134, 135, 136}

Plasminogen activator inhibitor-1

Plasminogen activator inhibitor-1 (PAI-1) is an inhibitor of fibrinolysis, the physiological process that degrades blood clots. Non-survivors of AP have higher concentrations of PAI-1 and d-dimer and lower activity of protein-C than do survivors.^{137, 138}

2.3 Genetic Factors in Pancreatitis**Familial pancreatitis**

Families with numerous members suffering from repeated episodes of pancreatitis are called familial pancreatitis families. Repeated acute episodes lead to fibrosis and calcifications of the gland. Research in familial pancreatitis has been successful in identifying genetic determinants related to pancreatitis. These findings can be applied to candidate genes in AP.

PRSSI

Hereditary pancreatitis (HP) is a rare form of recurrent AP and CP. A HP family was first described in 1952,¹³⁹ and recently one Finnish family was described.¹⁴⁰ The simple Mendelian model of inheritance of HP suggests that a single genetic defect is responsible for this disorder. With use of a genome-wide genetic linkage analysis, the gene was mapped to chromosome 7q35 in a large French family in 1996;¹⁴¹ this finding was concurrently confirmed by two other groups.^{142, 143} Subsequent candidate gene sequencing of the 7q35 chromosome region revealed a strong association of the c.365G > A (p.R122 H) mutation of the *PRSSI* gene encoding cationic trypsinogen with HP. Later, other mutations of this gene emerged in patients with hereditary or idiopathic CP. In vitro, these mutations lead to increased autocatalytic conversion of trypsinogen to active trypsin and thus probably cause premature intrapancreatic trypsinogen activation in vivo. The clinical presentation is highly variable, but most affected mutation carriers have a relatively mild disease;¹⁴⁴ however, these patients carry a risk for pancreatic cancer.¹⁴⁵ Mutations in *PRSSI* are rare among patients with alcoholic CP and in patients with idiopathic CP.¹⁴⁶

SPINK1

In 2000, Witt and colleagues first recognized the high incidence of mutations in the *SPINK1* gene in children and adolescents with CP.¹⁴⁷ In subsequent studies, the association between exonic *SPINK1* mutations, especially the allele called N34S, and idiopathic^{148, 149} and familial CP has been confirmed throughout the world. Homozygote carriers of the *SPINK1* N34S mutation are characterized by symptoms of CP in early childhood.¹⁵⁰ In several of these studies, the gene has been sequenced, with several variants described.^{151, 152} Furthermore, rare, clearly disease-causing mutations resulting in a complete functional loss of the protein have emerged.¹⁵³

Some studies on alcoholic CP revealed a less vital limited contribution by the exonic *SPINK* mutations to the disease.^{154, 155} The prevalence of the N34S and P55S mutations was 6.3% among patients with chronic alcoholic pancreatitis in the United States¹⁵⁶ and 2.4% in Korea.¹⁵⁷ In a report from India, the N34S mutation was recognized in 26.8% of patients with alcoholic CP.¹⁵⁸ The frequency of the N34S mutation in control populations has varied: 2.5% in Great-Britain, 2.8% in India, 1.6% in the United States and in Japan, and 0% in Brazil.¹⁵⁹ In a Finnish CP, sample prevalence of the N34S mutation was 12%.¹⁶⁰

The *SPINK1* gene is located on chromosome 5. The activity of the SPINK1 protein with the point mutation N34S is comparable to that of the wild-type protein; thus, the exact mechanism by which the N34S mutation is associated with pancreatitis remains unknown.¹⁶¹ Some speculate that haplotypes and intronic mutations associated with the N34S mutation are responsible for N34S being associated with decreased expression of *SPINK1*. Alternatively, increased degradation/inactivation by enzymes other than trypsin may be involved. In *SPINK1* knockout mice, the pancreas is absent. Because remnants of the pancreas appeared in some of the knockout mice, the mechanism has been suggested to be pancreatic autolysis.¹⁶²

CFTR

Cystic fibrosis gene (*CFTR*, cystic fibrosis transmembrane conductance regulator) mutations have been associated with CP. Cystic fibrosis, an autosomal recessive inherited disorder characterized by chronic obstructive pulmonary disease, also involves exocrine pancreatic insufficiency with maldigestion and increased sweat chloride concentration. Its incidence in the white population worldwide is about 1:2500. In Finland the disease is rare.¹⁶³ Several studies have found *CFTR* mutations in up to 30% of patients with idiopathic CP. *CFTR* mutations are associated with CP especially in patients with two or more heterozygous

mutations or one mutation accompanied by mutations of *PRSSI* and *SPINK1*^{164, 165} Penetrance of the identified pancreatitis-associated mutations is apparently incomplete.¹⁶⁶

Complex trait of acute pancreatitis

According to classical genetics, diseases are divided into Mendelian disorders and complex traits. While the former are attributed to single gene mutations with a simple inheritance, like familial pancreatitis, the latter are attributed to multiple genes, each playing a small and interactive role in susceptibility to the diseases. For complex traits, the contributions of most of the multiple etiologic factors are likely to be modest. In diseases, interaction with multiple genetic loci (locus, fixed position on a chromosome) and environmental factors is essential. Much heterogeneity exists, which means that different alleles (DNA coding that occupies a given locus) or loci cause disease in different groups. Incomplete penetrance is also a factor, meaning that not all susceptible individuals are affected.

In the case of AP, we have insufficient means to assess the amount of genetic load and heritability. Twin studies are one way to assess the impact of genetic predisposition to a disease.¹⁶⁷ In AP, however, such studies are unavailable. Furthermore, the heritability of alcohol-induced AP is undefined. In alcoholic pancreatitis, the environmental effect is essential in triggering the disease. Clinicians repeatedly ask, however, why only a sub-set of alcoholics develop alcoholic AP and CP. The answer is still undiscovered—but may associate with factors related to complex inheritance of the disease. Variation in a trait may be due to genetic variability in one group and to environmental variation in another.

The genetic factors involved in severe AP are likely to possess all the major features of complex traits. This includes additive effect of multiple minor components. The penetrance of each gene is influenced by gene-to-gene interactions and by interactions between the gene and the environmental and acquired factors. It is likely that several acquired conditions that are thus far unrecognized act as cofactors. It is likely that the allele frequency of the variations involved is relatively low in population. This coincides with the fact that the homozygote, with the special polymorphism/mutation, may be severely affected as AP interferes. Family trees are the only possible way to identify these rare variants. Protective genetic factors may also be responsible.

Defining the trait components

The classification of complex traits into well-defined categories, trait components, is the prerequisite for understanding the underlying pathogenetic mechanisms. Ideally, these trait components would be concordant with the genetic factors responsible for the phenotype. Poorly defined classification and phenotyping easily destroys all feasibility for a genetic study. In AP, this requires precise diagnosis and accurate classification of severity.

AP diagnosis must be definite. Inappropriate diagnosis is most often attributed to an overlooked differential diagnosis of hyperamylasinemia. Amylase values are easily elevated in patients with perforated peptic ulcer disease, intestinal obstruction or infarction, and in ruptured ectopic pregnancy, and postoperatively. The specificity of a serum amylase in determining AP can be increased by use of a cutoff of more than two to three times the upper limit of normal.¹⁶⁸ One of the frequent causes for hyperamylasinemia is renal insufficiency, which is associated with diminished excretion of amylase. Additionally, salivary gland lesions, various tumors (lung, esophagus, ovary, breast), pregnancy, burns, diabetic ketoacidosis, or drugs (morphine, codeine) may all cause hyperamylasinemia.

Differentiation between cases of AP and CP demands precision from the clinician. Diagnosis of CP is based on pancreatic exocrine or endocrine insufficiency or typical radiological signs of strictures in the pancreatic duct or calcifications in the parenchyma of the gland. Seldom diagnosis is made on histology specimens. Diagnostic tests for CP are not used routinely in emergency departments. Thus, inclusion of cases that have already developed CP easily interferes with assessment of patients with AP. Exclusion of CP patients can be done most strictly by including only those experiencing their first episode of AP—although few of these patients may have signs of CP in a thorough examination.

In patients with AP, different etiologies deserve their own categories. The alcoholic etiology is often easily detected by taking a proper history.¹⁶⁹ Degree of alcohol dependence can also be measured by validated questionnaires (The Alcohol Use Disorder Identification Test, AUDIT; The Short Alcohol Dependence Data, SADD). However, patients tend to overlook or lie about their use of alcohol, and it is obvious that some patients with a diagnosis of idiopathic AP suffer from alcohol-induced AP. Laboratory parameters (red blood cell mean corpuscular volume and disialotransferrin) are an additional aid for the physician in differentiating between alcohol and other etiologic factors. Biliary AP is associated with elevated liver values as well as dilatation in intra/extrahepatic bile ducts as signs of biliary stones in imaging studies. Idiopathic AP can be diagnosed in cases with no eligible etiologic diagnosis.¹⁷⁰ This means exclusion of alcoholic and biliary

etiologies, and rarer, but obvious etiologies for AP: familial pancreatitis, drugs, hypertriglyceridemia or hypercalcemia.

A definition of severe AP deserves some delineation for purposes of genetic studies. Widely used classifications like the Atlanta and Ranson serve as a sufficient basis regarding mild versus severe AP. The trait components in severe AP must, however, include identification of local and systemic complications separately. The systemic complications include the distant OFs occurring early during the disease (during the first week). Early OF may to be associated with more advanced local complications and a complicated clinical course. Early OF that is continuous is associated with mortality, and must be defined as a trait component as such. OF occurring later represent another phenomenon, and are closely associated with infectious complications locally and around the pancreas. Local complications with infection are associated with late mortality. Individual susceptibility to infectious complications in necrotic foci in and around the pancreas may in part be genetically determined. The late infectious complications are important, since these contribute to AP mortality.

Candidate gene studies

The scientific community has shown interest in genetic determinants of disease severity in AP since the early 1990s.¹⁷¹ Table 2 presents the case-control studies including patients with AP.¹⁷²

Genetic studies in chronic pancreatitis

Idiopathic CP is the leading type of CP in children and in nonalcoholic adults. The risk for developing it is higher in individuals who have mutations of the *CFTR* and of *SPINK1* genes. In studies from the United States and France, risk for idiopathic CP is increased about 40-fold from having two abnormal copies of the *CFTR* gene, about 14-fold from having the N34S *SPINK1* mutation, and about 500-fold by having both. When idiopathic CP patients have two abnormal copies of the *CFTR* gene, there is also evidence of reduced residual *CFTR* protein function in extrapancreatic tissues based on clinical findings and nasal ion transport responses.¹⁷³ The human major histocompatibility complex (MHC) is a group of genes residing on chromosome 6 which code for the human leukocyte antigen system (HLA). This region was early recognized to have influence on inflammatory processes. Numerous investigations from the 1980s reported dissimilar associations between different HLA subtypes and CP.^{174, 175, 176, 177, 178, 179}

A summary of genetic case-control studies in CP is in Table 3.

2.4 Genetic Factors in Systemic Inflammation

The markedly different responses of seemingly similar individuals to the same inflammatory or infectious agents has attracted notice. The death from infection of a biological parent is associated with a five-fold greater risk of death from infection in adoptees.¹⁸⁰ For a seemingly adverse mutation to be preserved in the human genome, a survival advantage in another single disease, is a prerequisite.¹⁸¹

Cytokine gene polymorphism

Genes coding for cytokines are important candidate genes for determining the strength of a individual's response to injury. In cells, genes consist of a long strand of DNA that contains a promoter—which controls the activity of a gene—and a coding sequence, which determines the gene products. Study of polymorphisms in the areas of gene promoters of cytokine genes has exploded during the past ten years.^{182,183,184,185,186} Associations may exist between specific polymorphisms and ischemic heart disease,¹⁸⁷ sepsis and septic shock,¹⁸⁸ surgical injury in aortic repair,¹⁸⁹ and psoriasis.¹⁹⁰

Hemostatic gene polymorphism

The importance of hemostatic gene polymorphism for the clinical picture of an infectious disease was pointed out in 1999 by investigators of meningococcal disease.¹⁹¹ Patients carrying the *PAI-1* 4G/4G prothrombotic genotype had higher mortality. These results were repeated by another group some years later.¹⁹² The *PAI-1* gene has also been associated with a prognosis of pneumonia and cerebrovascular disease.^{193, 194}

Table 2. Studies of genetic factors in acute pancreatitis (AP).

Gene	Investigator ^{reference} year	AP, N	Severe AP, n	Classification of severe AP	Major finding, remarks
<i>ADH2, ADH3, ALDH2, P4502E1</i>	Chao ¹⁹⁵ 1997	48	Not stated	Not stated	<i>ADH2*2</i> variant may influence susceptibility to acute alcoholic pancreatitis.
<i>TNF</i>	Sagen ¹⁹⁶ 2000	135	97	Atlanta	Variants -308 A/G do not associate with severe AP.
<i>IL1, IL-1RN, IL-1B</i>	Smithies ¹⁷⁰ 2000	116	Not stated	Not stated	Variants of <i>IL-1RN</i> appear to determine severity of AP and susceptibility to idiopathic AP.
<i>TNF, IL-1, IL-1RA</i>	Powell ¹⁹⁷ 2001	190	113	Atlanta	No association.
<i>TNF</i>	Zhang ¹⁹⁸ 2003	208	102	Apache >8 or CT severity index >4	Allele -308A strongly associates with early septic shock in AP.
<i>ADH2, ADH3, ALDH2, P4502E1</i>	Chao ¹⁹⁹ 2003	92	Not stated	Not stated	<i>ADH2*1</i> and <i>ALDH2*2</i> appear to differ in disease-specified subpopulations of alcoholics.
<i>CD14</i>	Rahman ²⁰⁰ 2004	117	34	Atlanta	No association.

<i>GSTT-1, MnsOD, Catalase</i>	Rahman ²⁰¹ 2004	320	90	Atlanta	The functional <i>GSTT-1</i> * A genotype was associated with severe attacks of pancreatitis.
<i>IL-1β, IL-10, CD14</i>	Zhang ²⁰² 2005	215	109	Apache >8 or CT severity index >4	Allele -1082G associates with septic shock in AP.
<i>CD14</i>	Chao ²⁰³ 2005	100	Not stated	Not stated	Allele -159C associates with alcoholic AP compared to controls with other alcohol-induced complications.
<i>TNF, HSPA1B, CD14</i>	Balog ²⁰⁴ 2005	77	48	Ranson	<i>TNF</i> : moderate increase in A allele in severe AP patients (p=0.046). <i>HSPA1B</i> : G allele has a risk, OR: 5.7; 95% CI = 2.0-16.1.
<i>MCP-1</i>	Papachristou ²⁰⁵ 2005	77	14	Ranson	Allele -2518G associates with severe AP.
<i>IL-8, TLR4</i>	Hofner ²⁰⁶ 2006	92	38	Ranson	<i>IL-8</i> -251 A allele associates with severe AP.
<i>GSTT-1</i>	Bhat ²⁰⁷ 2006	91	19	Ranson	No association.
<i>MIF</i>	Makhlaja ²⁰⁸ 2007	167	Not stated	Not stated	Allele -173 C associates with AP.
<i>HFE</i>	Huei T. ²⁰⁹ 2007	34	Not stated	Not stated	No association.
<i>TLR4</i>	Gao ²¹⁰ 2007	115	115	All had necrosis in pancreas assessed by CT	<i>TLR4</i> 896A>G is a potential risk for infected necrosis.

Table 3. Studies of genetic factors in chronic pancreatitis (CP) excluding studies with SPINK 1 and trypsinogen and CFTR genes.

Gene		Investigator ^{reference} year	Number of studied			Major finding, remarks
Name	Product, function		SNPs	patients	controls	
<i>CYP2E1</i>	Alcohol metabolism	Yang ²¹¹ 2001	1	38	38	No association.
<i>IL10, TNF</i>	Cytokines	Beranek ²¹² 2003	6	335	141	Variant <i>TNF-238A</i> may be a risk factor in hereditary pancreatitis.
<i>Keratin 8</i>	Components of the intermediate filament cytoskeleton of pancreatic acinar cells	Cavestro ²¹³ 2003	1	68	100	Glycine-to-cysteine mutations at position 61 found more often (8.9% vs. 0%, p<0.003) in patients.
<i>TNF, TNFR1</i>	Cytokine, receptor of TNFa	Schneider ²¹⁴ 2003	3	54	0	No association.
<i>TNF, TGFβ1, IL10 Interferon-γ</i>	Activation of collagen synthesis in pancreas, cytokines	Schneider ²¹⁵ 2004	7	140	73	No association.
<i>ACE</i>	Angiotensin converting enzyme	Oruc ²¹⁶ 2004	1	155	163	No association.
<i>UDP-glucuronosyltransferases</i>	Detoxification enzymes	Verlaan ²¹⁷ 2004	4	258	140	No association.
<i>GSTM1, GSTT1, GSTP1, CYP2E1, CYP1A1</i>	Detoxification enzymes	Burim ²¹⁸ 2004	5	14	221	Association between occurrence of Val/Val <i>GSTP1</i> genotype and CP.
<i>GSTT1, GSTM1, GSTP1, MnSOD, Catalase</i>	Antioxidant enzymes	Rahman ²¹⁹ 2005	5	122	245	<i>GSTT-1</i> *A genotype associates with idiopathic CP.

<i>PON1</i>	Antioxidant enzyme	Verlaan ²²⁰ 2005	2	136	206	<i>PON1</i> -192Q allele may be associated with idiopathic CP.
<i>IL-1β, IL-6, IL-8, TNF, VEGF, TGFβ1, ICAM-1</i>	Cytokines	Howell ²²¹ 2005	8	53	266	Promoter polymorphisms of <i>IL-8</i> and <i>ICAM</i> may be associated with CP.
<i>TGF-β1</i>	Activation of collagen synthesis in pancreas	Bohm ²²² 2005	3	130	100	No association.
<i>Keratin 8</i>	Components of the intermediate filament cytoskeleton in pancreas	Schneider ²²³ 2006	1	288	271	No association.
<i>MCP-1</i>	Monocyte chemotactic protein	Sass ²²⁴ 2006	1	177	116	No association.
<i>CTLA4, TNF</i>	T-cell immune regulation, cytokine	Chang ²²⁵ 2007	6	124	200	Increase in <i>CTLA4</i> 49A carriers in patients with autoimmune pancreatitis.
<i>Cathepsin B</i>	Lysosomal cysteine proteinase	Weiss ²²⁶ 2007	1	64	100	No association in idiopathic CP.
<i>ADH2, ADH3, ALDH2</i>	Ethanol metabolism	Cichoż-Lach ²²⁷ 2007	3	44	97	<i>ADH3</i> *1/*1 genotype associates with alcoholic CP.
<i>ADH2, ALDH2</i>	Ethanol metabolism	Shimosegawa ²²⁸ 2007	6	156	165	<i>ADH2</i> *2 allele associates with alcoholic CP
<i>MnSOD, GSTP1</i>	Antioxidative capacity	Osterreicher ²²⁹ 2007	2	165	300	No association. Focus on alcoholic CP
<i>HFE</i>	Iron metabolism (hemochromatosis)	Huc1 ¹⁹⁰ 2007	2	756	781	No association. Focus on idiopathic CP.
<i>TGFβ1, IL-8, TNF</i>	Cytokines	Farkas ²³⁰ 2007	3	83	75	Correlations of the <i>TGF-β1</i> and <i>IL-8</i> SNPs and CP were found.

The Leiden mutation of *FV* is a procoagulative mutation with a population prevalence of 3% to 5%. *FV* Leiden is a risk factor for deep venous thrombosis, but may be advantageous in severe infections.²³¹

Genetic determinants of coagulation and fibrinolysis are candidate genes in sepsis²³² and in lung injury.²³³ Study of the importance of these polymorphisms in coronary heart disease has been extensive, but their influence is marginal.²³⁴

Adipokines

Obesity has a strong hereditary component.²³⁵ Adipokines are fat-derived hormones and responsible for metabolic effects of obesity such as low-grade systemic inflammation. The most well-known adipokines are adiponectin, identified in 1996 and leptin, identified in 1994.

In obesity adiponectin is downregulated. Several clinical studies demonstrate the inverse relationship between plasma adiponectin levels and several inflammatory markers including CRP.²³⁶ Adiponectin attenuates inflammatory responses to multiple stimuli by modulating signaling pathways in a variety of cell types. The plasma level of adiponectin is, in part, genetically determined.^{237, 238} The latest studies in homozygotic twins do support this.²³⁹ The *APMI* gene, the gene encoding adiponectin, has been suggested to be an important determinant of adiponectin levels.^{240, 241, 242, 243, 244}

In obesity leptin is upregulated. In contrast to adiponectin, leptin has proinflammatory properties and may enhance systemic inflammatory reactions.^{245, 246, 247} It is encoded by the *Ob* gene. The receptors for leptin are more tightly genetically determined than are those for plasma levels of leptin.²⁴⁸ Leptin levels are, however, also, in part, genetically determined.²⁴⁹

3 PRESENT INVESTIGATION

3.1 Aims of the Study

The purpose of the present study was to identify genetically determined prognostic factors in AP.

The specific aims of the study were to:

1. Evaluate the relevance of *SPINK1* gene mutations N34S and P55S in AP patients.
2. Discover whether polymorphisms of the inflammatory genes *CD14*, *TNF*, *IL-10*, and *HSPA1B* are associated with severe AP and alcohol-induced AP.
3. Discover any association between the procoagulative hemostatic gene polymorphisms *PAI-1* 4G/5G and *FV* Leiden and severe AP.
4. Define whether adipokines, with their capacity to modify systemic inflammation, are associated with severe AP.

3.2 Patients and Methods

Patients with acute pancreatitis

Study I: 324 patients were included between September 1998 and April 2003 at the emergency unit of the Helsinki University Central Hospital. Additionally, a retrospectively recruited group had severe AP during the 1990s, and this group comprised 47 patients. These patients were contacted by letter invitation and volunteered for blood sampling.

Studies II & III: This prospectively collected group comprised 349 AP patients admitted to the Helsinki University Central Hospital Emergency Unit during the years 1998 to 2003. A retrospective group of 48 patients with severe AP during the 1990s was available from Study I.

Study IV: The study population comprised 410 patients admitted to the Helsinki Central Hospital from April 1999 to January 2004. Because adipokine levels are associated with gender and body mass index (BMI), a matched case-control design was used. Matching included gender, age (± 10 years), BMI ($\pm 3 \text{ kg/m}^2$) and etiology of AP. A total of 12 pairs could be identified among the 410 patients with AP.

The study protocols were approved by the local institutional ethics committee. The patients gave their informed consent for participation in the study.

Table 4. Acute pancreatitis (AP) patients in Studies I, II, III and IV.

Study	Number of study patients				
	Total number	Men	Severe AP	Organ failure	Alcoholic AP
I	371	257 (69%)	164 (44%)	52 (14%)	229 (62%)
II	397	284 (72%)	152 (38%)	55 (14%)	214 (54%)
III	397	284 (72%)	152 (38%)	55 (14%)	214 (54%)
IV	24	16 (67 %)	12 (50%)	8 (33%)	14 (59%)

Controls

Study I: 459 blood donors served as controls (The Finnish Red Cross, Helsinki, Finland).

Studies II & III: A total of 310 individuals served as controls; 218 were from the WHO/ISBRA Multicenter, Multinational Study on the Trait and State Markers of Alcohol Consumption and Alcoholism²⁵⁰ and comprised Finnish men with detailed data of their alcohol consumption. Of these 218 men, 70 consumed alcohol amounting to more than 40 g/day; these formed a subgroup of controls denoted as heavy drinkers. Of the 218 controls, 59 were non-drinkers, and 89 consumed <40 mg alcohol per day. Additionally, 92 blood donors (The Finnish Red Cross, Helsinki, Finland) provided blood samples as controls. All the controls and the cases were Caucasian and originated from the comparable geographic area of Finland—Uusimaa.

Study IV: Patients with mild AP served as matched controls for patients with severe AP.

Definitions of phenotypes

Diagnosis: AP was diagnosed if the patient had a clinical presentation consistent with AP and a plasma amylase activity greater than three times the upper reference limit, or confirmation of AP by CT in patients with normal or marginally elevated amylase concentrations. Abdominal pain, increased tenderness, rebound, distension, vomiting, fever, tachycardia, bowel paralysis, Grey Turner's sign or Cullen's sign were considered clinical signs of AP.

Alcohol-induced AP: When no other etiology was obvious and the patient admitted use of alcohol during the week previous to hospitalization, the AP was designated as alcohol-induced.

Biliary AP: When biliary stones were detected in ultrasound examination the AP was designated as biliary.

Idiopathic AP: After diagnosis of AP, possible etiologic factors were assessed (alcohol, biliary stones, medication, tumors, hypertriglyceridemia, hypercalcemia). If no such causative factor could be identified, the AP was designated as idiopathic.

Severe AP: Severity of AP was categorized by the clinically based classification of the Atlanta symposium 1993.²⁴ According to this classification, mild AP is associated with minimal organ dysfunction and an uneventful recovery. If systemic or local complications or both are present, AP is classified as severe.

OF: OF is consistent with need for mechanical ventilatory support or with hemodialysis due to renal failure. Vasopressors were used according to current clinical practice. Criteria for initiating mechanical ventilation were tachypnoe with respiratory rate over 35/minute or need for inspiratory oxygen fraction >0.6 in order to maintain arterial partial pressure of oxygen >8 kPa. Hemodialysis was started in patients with significant reduction in renal function indicated by increased concentrations of plasma creatinine (>300 mmol/l) or plasma urea (>40 mmol/l) and progressive metabolic asidosis (pH<7.28).

Infectious complication: Positive blood culture from wounds, urine or blood during the hospitalization for AP. In rare cases, culture samples were taken peroperatively. Fine-needle puncture samples from the intra-abdominal abscesses and necroses were collected when appropriate.

Laboratory methods

DNA isolation methods

DNA from the whole blood samples of patients and blood donor controls was extracted with a QIAamp DNA isolation kit (Qiagen, Valencia, CA, USA). DNA samples from the WHO/ISBRA Multicenter, Multinational Study on the Trait and State Markers of Alcohol Consumption and Alcoholism (218 controls, Study II & III) had been isolated by the usual phenol-extraction method.

Genotyping methods

Study I: The mutations were detected by a solid-phase minisequencing using ³H-labeled nucleotides. After PCR amplification, 10 µL of the PCR product was captured in streptavidin-coated scintillating microtitration plate wells (EG&G Wallac, Turku, Finland) with 40 µL of buffer (0.15 mol/L NaCl, 20 mmol/L Na-phosphate, pH 7.4, and 0.1% Tween-20) per well. These samples were incubated for 1 hour at room temperature with gentle shaking, after which the plate was washed four times with a buffer containing 40 mmol/L Tris-HCl (pH 8.8), 1 mmol/L EDTA, 50 mmol/L NaCl, and 0.1% Tween-20 with an automatic microplate washer (Tecan 96 PW; Tecan Nordic AB, Mölndal, Sweden). The bound PCR products were

denatured with 100 µL of 50 mmol/L NaOH for 5 minutes at room temperature. The wells were washed four times with the washing buffer.

The minisequencing reaction mixture, containing, in separate wells, detection step primers for the N34S (5'-TTAGGCCAAATGTTACA-3') and P55S (5'-GGACTGATGGAAATACTTAT-3') mutations and wild-type alleles at 0.2 µM concentration, the appropriate H dNTPs (Amersham Biosciences Europe, Espoo, Finland) at 0.02 µmol/L concentration, and 0.5 U of Dynazyme DNA polymerase in 100 µL of 1× PCR buffer, were added to wells. The wells were incubated at 55°C for 15 minutes with gentle shaking and washed four times with washing buffer. The incorporated radioactivity was measured in a MicroBeta counter (EG&G Wallac) and expressed as counts per minute. The minisequencing reaction was performed in two parallel reactions with all four nucleotides for each sample. Results for each sample with a mutation was confirmed by direct sequencing by purifying the PCR products using a kit for DNA extraction (Amicon Ultrafree-DA; Millipore Corp., Bedford, MA, USA) and sequencing with the ABI Prism Dye Terminator Cycle Sequencing Core Kit with AmpliTaq Polymerase and the ABI Prism 310 Genetic Analyser (PE Biosystems, Foster City, CA, USA).

Studies II & III: Samples were genotyped by a matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF) mass spectrometry-assisted genotyping method (Sequenom, San Diego, CA, USA). *TNF* promoter -308 G/A, *CD14* promoter -159 C/T, and *HSPA1B* +1267 A/G, and *IL-10* -592 and -1082 genomic sequences were obtained from the SNP consortium database (<http://www.ncbi.nlm.nih.gov>). PCR assays and associated reactions were designated with Spectro DESIGNER software (Sequenom), and primers came from Metabion (Planegg-Martinsried, Germany) (Table 5).

All amplification reactions were run in a total volume of 5 µl with 2.5 ng of genomic DNA, 1 pmol of each amplification primer, 0.2 mM of each dNTP, 2.5 mM MgCl₂, and 0.2 U of HotStarTaq DNA polymerase (Qiagen). Reactions were heated at 95°C for 15 min, subjected to 45 cycles of amplification (20 s at 94°C, 30 s at 60°C, 30 s at 72°C) before final extension of 10 min at 72°C. Extension reactions were conducted in a volume of 2 µl, using 5 pmol of allele-specific extension primer and the Mass EXTEND Reagents Kit, before being cleaned with SpectroCLEANER (Sequenom) on a MULTIMEK 96 robot (Beckman Coulter, Fullerton, CA, USA). Cleaned products were loaded onto a 384-element chip with a nanoliter pipetting system. (SpectroCHIP, SpectroJet, Sequenom), analyzed by a MassARRAY mass spectrometer (Sequenom /Bruker Daltonik, Bremen, Germany), and peaks were identified with the SpectroTYPER RT 2.0 software (Sequenom).

Table 5. Database single nucleotide polymorphism identity numbers (DbSNP) and primers used for MALDI-TOF determination of polymorphisms.

Gene, SNP	DbSNP	5' capture primer	3' capture primer	Extend primer (5'→3')
TNF -308	rs1800629	ACGTTGGATGGATTGTGTGT AGGACCCTG	ACGTTGGATGGGTCCCCAAAA GAAATGGAG	AATAGGTTTTGAGGGGCATG
HSPA1B +1267	rs1061581	ACGTTGGATGCATCCCCAAGG TGCAGAAGCT	ACGTTGGATGGGGTTGATGCT CTTGTCAG	CGCCCGTTGAAGAAGTC
CD14 -159	rs2569190	ACGTTGGATGGCAATGAAGGA TGTTTCAGG	ACGTTGGATGAGACACAGAAC CCTAGATGC	CAGAATCCTTCCTGTTACGG
IL-10 -592	rs1800872	ACGTTGGATGAAAGGAGCCTG GAACACATC	ACGTTGGATGTCCTCAAAGTT CCCAAGCAG	CCAGAGACTGGCTTCCTACAG
IL-10 -1082	rs1800896	ACGTTGGATGGACAACACTAC TAAGGCTTC	ACGTTGGATGATTCCATGGAG GCTGGATAG	ACCTATCCCTACTTCCCC
PAI-1 4G/5G	rs1799889	ACGTTGGATGCTCTTGGTCTTT CCCTCATC	ACGTTGGATGCACAGAGAGAG TCTGGACAC	AGTCTGGACACGTGGGG
FV Leiden	rs6025	ACGTTGGATGACCTAACATGT TCTAGCCAG	ACGTTGGATGTCTGTAAGAGC AGATCCCTG	AGAGCAGATCCCTGGACAGGC

ELISA measurements of adipokines

Study IV: ELISA (Enzyme-Linked ImmunoSorbant Assay) -based methods, Quantikine Human Adiponectin, and Quantikine Human Leptin Immunoassays (R&D Systems, Minneapolis, MN, USA), were used to measure adipokine levels. The ACD plasma samples, stored at -80°C, were diluted 100-fold, as recommended by the manufacturer. The analyses were performed in a blinded fashion.

Statistical methods

Statistical significance between the groups was tested with the X^2 Chi squared test and Fisher's exact or alternatively the Mann-Whitney U-test when appropriate. $P < 0.05$ was considered statistically significant.

In pretest power analysis for the mutations studied, the prevalence of a mutation of *SPINK1* was assumed to be 2% in the population and 7% among the patients. The estimate of frequency in the control population was based on previous studies, and the frequency in patients was estimated to be one-half of the frequency of the mutations found in patients with CP. A sample size of 360 individuals in each group was estimated to be sufficient to produce statistically (power 90%, significance 0.05) significant results.²⁵¹

The power analysis for sample sizes in populations with unknown genotype frequencies is complicated.²⁵² When considering the allele frequencies, a sample size of 360 differences that are smaller than 10% between study groups will not be considered statistically significant.

3.3 Results

SPINK1 mutations (Study I)

The exonic *SPINK1* gene mutation N34S occurred in 29 (7.8%) patients with AP and in 12 (2.6%) controls ($p < 0.001$, Fisher's exact test). The P55S mutation was found at a frequency of 0.8% among patients with AP and 1.3% among the controls. None of the patients or controls was found to carry both the N43S and P55S mutations. The median age of patients with the N34S mutation was 45 years (range 20-73) and was 49 years (range 20-94) in those without mutations ($p = 0.24$, Mann-Whitney U-test). One patient, a 28-year-old male with idiopathic mild AP, but none of the controls was found to be homozygous for the N34S mutation

Inflammatory gene polymorphisms (Study II)

The allele frequencies of the *TNF*, *HSPA1B*, *CD14* and *IL10* polymorphisms did not differ significantly between AP patients and controls (Table 6). These results were in Hardy-Weinberg equilibrium (HWE), with the exception of the *CD14* gene in the control group of 218 samples from the WHO/ISBRA Multicenter, Multinational Study on the Trait and State Markers of Alcohol Consumption and Alcoholism. The

genotyping was repeated for the *CD14* locus for all patients and controls, and the results were identical with the initial genotyping.

Infectious complications occurred in 47 patients, and these had a genotype distribution not differing from that of those with mild, uncomplicated disease. The genotype distribution did not differ significantly between patients with alcohol-induced AP and the control group of heavy drinkers. Furthermore, in the patients with biliary AP and alcohol-induced AP the major allele frequency was similar.

Haplotypes of the *IL-10* promoter were identified as described by Warle.²⁵³ As perfect linkage disequilibrium exists with *IL-10* -592 and *IL-10* -819, the “high producer” haplotype is GC, and the “low producer” haplotype is AT, based on genotyping results of locuses *IL-10* -1082 and *IL-10* -592. We could identify 70 patients as homozygous (GC/GC) for the “high producer” haplotype. These patients were evenly distributed among those with mild (50; 21% patients) and severe AP (20; 20% patients). Similarly, the patients with homozygosity for the “low producer” haplotype (AT/AT) were evenly distributed among those with mild AP (13; 5%) and severe (5; 5%) AP.

Hemostatic gene polymorphisms (Study III)

No deviation from HWE could be detected in the genotyping results for *FV* Leiden and of *PAI-1* 4G/5G genotypes.

The genotype distribution in *PAI-1* 4G/5G polymorphism between mild and severe AP differed. The prothrombotic allele 4G was underrepresented in patients with severe AP, $p < 0.05$. Among those 55 AP patients with OF, the allele frequency for 4G was 0.49; 21 patients were homozygotes, and 30 heterozygotes for 4G allele in patients with OF. Of 47 patients with infectious complications, homozygotes for the 4G mutation were 11 (23.4%) and heterozygotes 28 (59.6%); with an equal allele frequency of 0.53 for 4G in this patient group.

The patients with the 4G/4G genotype had a shorter hospital stay than did those carrying the 5G/5G genotype ($p < 0.02$, Mann-Whitney U-test). Patients who were homozygote carriers for 4G had hospital a stay median 6 days (range 1–155), and patients who were homozygote carriers for 5G had median a hospital stay of 10 days (range 1–105). Among those with mild AP, a similar trend was evident ($p < 0.09$, Mann-Whitney U-test). The median hospital stay for 4G/4G mild AP was 5 days (range 1–16) and for 5G/5G genotypes a median 6 days

(range 1-24). The length of hospital stay was a median 5 (range 1-24) days for those with mild AP and for patients with severe AP without OF it was 12.5 (range 1-110) days, significantly longer ($p<0.0001$, MannWhitney U-test). For those with OF the median hospital stay was 43 (range 2-172) days, significantly longer than those with severe AP without OF ($p<0.0001$, Mann-Whitney U-test). The allele frequency for 4G was 0.54 among those with alcohol-induced AP and 0.46 for those with biliary AP ($p>0.05$).

The genotype distribution in the *FV* Leiden mutation did not differ significantly between mild and severe AP. Furthermore, the genotype distributions of the subgroup of the patients with OF and patients with infectious complications did not differ from those of patients with mild AP. Of the 47 patients with infectious complications, one (2.1%) was detected to be a carrier of the Leiden mutation. The Leiden mutaton was detected in six (2.8%) patients with alcohol-induced AP and in two (2.5%) of biliary AP patients. Carriership of the Leiden mutation did not affect length of hospital stay. Patients carrying the Leiden mutation had a median hospital stay of 8 days (range 5-79), and patients without it had a median hospital stay of 7 days (range 1-172), ($p>0.05$, Mann-Whitney U-test)

Concordant occurrence of prothrombotic polymorphisms, Leiden mutation, and *PAI-1* 4G was found in ten cases of AP. Four patients were among those with mild AP and six among those with severe AP, two of whom had infectious complications, and one had OF.

Adipokine levels (Study IV)

The on-admission adiponectin values were a median 5 642 ng/mL (range 1 201-19 400 ng/mL) for the severe AP group and median 6 314 ng/mL (range 1 980-24 340 ng/mL) for the mild AP group ($p>0.05$). The values of the nine patients verified to have pancreatic necrosis were a median 5 495 ng/mL (range 1 201-18 822 ng/mL). Their adiponectin levels remained stable during follow-up in patients with mild AP (Figure 2), but in patients with severe AP seemed to vary. Maximum variation in adiponectin level (the highest value minus the lowest value) was greater in severe AP (2 456 ng/mL; range 1 270-8 617 ng/mL) than in mild AP, 546 ng/mL (range 0-2 730 ng/mL), ($p=0.001$).

Table 6. Genotyping results in Studies I, II and III with allele frequencies of risk genotypes in different study groups.

Gene, allele	Successful genotyping, n (%)	Allele frequency in the study group				
		Controls, all	AP, all	Severe AP	AP with OF	Alcohol-induced AP
<i>SPINK1</i> N34S	830 (100%)	2.6%	7.8% [§]	9.1%	11.5%	9.1%
<i>SPINK1</i> P55S	830 (100%)	1.3%	0.8%	0.6%	0.0%	1.3%
<i>TNF</i> -308A	703 (99.4%)	13.7%	12.3%	12.5%	14.5%	12.3%
<i>HSPA1B</i> +1267G	689 (97.5%)	50.8%	52.1%	50.7%	50.0%	52.2%
<i>CD14</i> -159T	705 (99.7%)	36.1%	39.9%	41.1%	37.6%	41.7%
<i>IL-10</i> -592T	703 (99.4%)	23.1%	23.4%	25.3%	27.2%	25.0%
<i>IL-10</i> -1082A	697 (98.6%)	47.1%	43.5%	42.1%	38.8%	43.0%
<i>PAL-1</i> 4G	697 (98.6%)	54.4%	53.4%	48.3%	50.0%	53.8%
<i>FV</i> Leiden	700 (99.0%)	2.1%	1.7%	1.7%	2.7%	1.4%

[§] Significantly different from controls, p<0.001, Fischer's exact test.

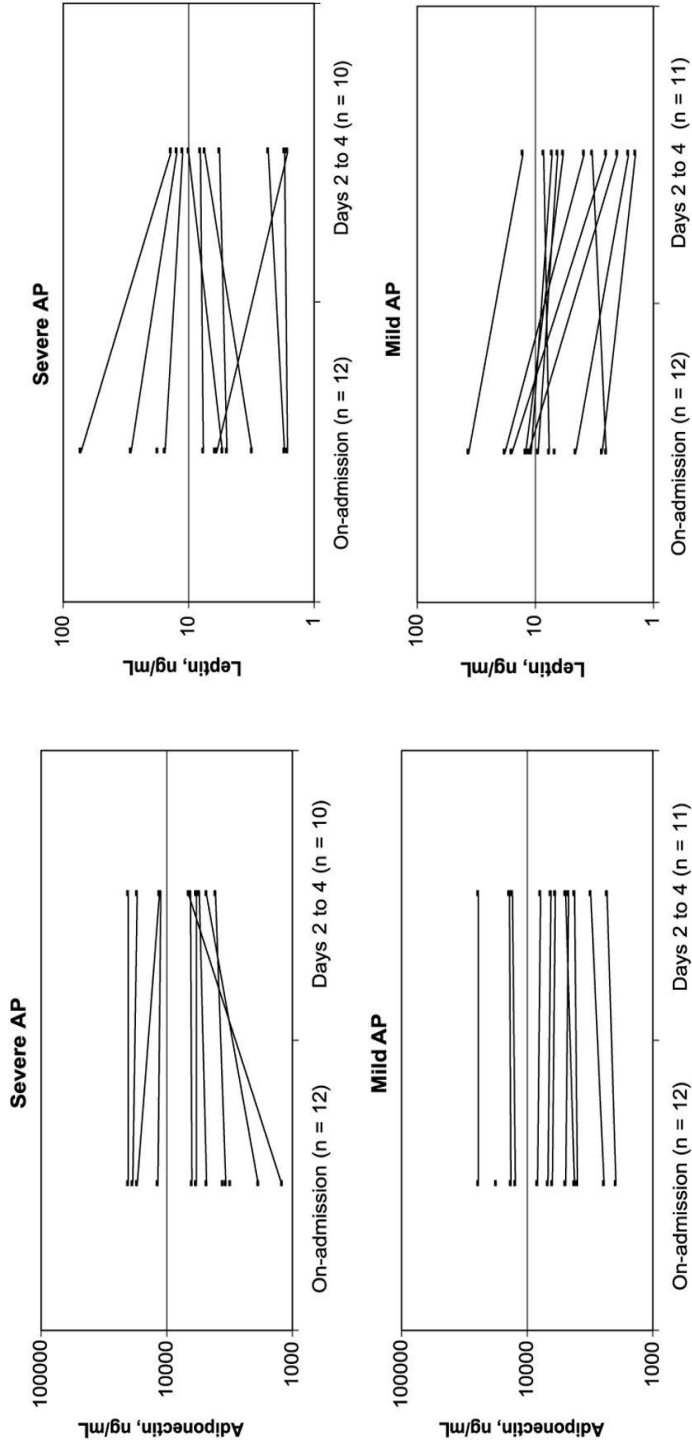


Figure 2. Adiponectin and leptin levels in patients with mild and severe AP. In AP patients matched by age, gender, body mass index, and etiology, the on-admission plasma levels of adiponectin and leptin did not correlate with disease severity, suggesting that they do not affect the course of AP.

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The leptin values on admission were median 6.1 ng/mL (range 1.6-72.9 ng/mL) in the severe AP group and median 9.0 ng/mL (range 2.5-36.5 ng/mL) in the mild AP group ($p>0.05$). The peak values (median) were 12.6 ng/mL (range 2.1-72.9 ng/mL) for severe AP and 10.6 ng/mL (range, 2.7-36.3 ng/mL) for mild AP ($p>0.05$). The nine patients who were verified to have pancreatic necrosis had on-admission leptin levels (median) of 6.0 ng/mL (range 1.6-72.9 ng/mL). In the mild AP group (Figure 2), the on-admission value was significantly higher than the respective 2 to 4 days' value ($p=0.005$). In the severe AP group, the values on admission and at days 2 to 4 did not differ significantly ($p>0.05$).

3.4 Discussion

Methods

The aim of a genetic study is to identify heritable disease-related genotypes, mutations, phenotypes, or karyotypes. Twin studies and family studies with linkage analysis are powerful study settings for genetic studies. In AP the case-control setting with association analysis is available due to the complex nature of the disease.

The relative risk of a genotype affects sample-size requirements. Furthermore, disease allele frequency affects minimum sample size requirements. Higher allele frequency is associated with larger sample size requirements in order to reveal statistical associations.²⁵⁴ In AP, the relative risk of any genotype is likely to be modest, and sample group size requirements are higher than the sizes available. Our study population is relatively large compared to other patient samples reported thus far. However, because we study a multifactorial disease, the patient population should be over 1000. As our main interest is severe AP and possibly more particularly OF accompanying severe AP, our patient sample can be considered modest.

Here we present the results of the thus-far largest case-control association study of AP and inflammatory gene polymorphisms. The study involved 397 patients, of whom 154 had severe AP, and of those, 55 patients had OF, the feature closely associated with mortality from AP. Our retrospectively collected patient group is subject to some problems concerning the most seriously affected individuals, not included because they were dead or disabled, unable to participate in voluntary blood sampling. However, the retrospective group included only patients with severe AP according to the criteria of Atlanta, and as high as 40% of them had OF. Thus, the retrospective patient group included patients with very severe disease.

A case-control study setting is sensitive to multiple confounding factors like population stratification.²⁵⁵ In our study, the control populations were drawn from the Metropolitan Helsinki Area (Uusimaa), the same area from which patients are allocated to the Helsinki University Hospital, as we aimed at minimizing population stratification. Furthermore, all the study subjects were Caucasian. The Finnish WHO/ISBRA study subjects represented a group with clearly defined alcohol consumption and no pancreatic disease. As to blood donors, it was unknown whether they had pancreatitis, and furthermore, their lifestyle may have been protective against AP compared to that of the general population.

We had the opportunity to focus on alcohol-induced AP because its incidence in Finland is extremely high. Due to this incidence, in this population the identification of genetic factors may indeed be possible. It is also possible that the Finns' high incidence of alcoholic pancreatitis is related more to their binge-drinking pattern and to diet. However, if such a genetic factor exists, Finns are a good population in which to study it. In the metropolitan area of Helsinki, Uusimaa, the population is not as isolated as in remote provinces and Lapland. Those population isolates would be even more ideal for studying complex traits.²⁵⁶

The subgroup defined as heavy drinkers consumed alcohol at more than 40 g/day. This amount of alcohol, by definition, is not high. Ideally, the control group would have been people consuming even higher amounts of alcohol and being examined for pancreatic disease. Selection of a well-matched control group is of high importance in genetic case-control studies.²⁵⁷

Misclassification errors are possible in genotypes and in phenotypes and cause loss of study power. We dissected the trait components of severe AP: OF and infectious complications. Furthermore, we identified the alcoholic etiology. The definition of alcoholic AP could have been made more strictly by employing disialotransferrin measurements or formulating inquiries into alcohol consumption. We do not know whether our definitions of trait components are optimal, and the researcher has to be open to new ideas of dissecting complex traits.^{258, 259}

As to our genotyping procedure, our results can be considered reliable. Only a small number of samples (1.1%) were not genotyped. We repeated the genotyping of *CD14* polymorphism, and no misclassifications were detectable. For case-control data, duplicate samples can be genotyped, and controls are tested for deviations from HWE. Duplicate samples can provide accurate estimates of genotyping error rates, unless systematic genotyping errors have occurred. Although genotyping errors can cause deviations from HWE, these deviations are usually small, and the power to detect them is low except for high rates of genotyping error or large sample sizes, or both.²⁶⁰

The matched case-control design in Study IV provides an efficient method of controlling for major confounders.

Statistical methods were adopted according to the principal rules. The chi squared X^2 test and Fisher's exact or alternatively the Mann-Whitney U-test were used when appropriate.

SPINK1

This is, to our knowledge, the first report of *SPINK1* gene mutations in AP and shows a surprisingly high prevalence (7.8%) of the N34S mutation of the *SPINK1* gene in 371 patients. Our finding suggests a link between *SPINK1* mutations and AP: The *SPINK1* N34S mutation was associated not only with a particular type of CP but may also be a risk factor for pancreatitis in general. The frequency of the N34S mutation in the control population of 2.6% is comparable with that in other studies. No association emerged between the other *SPINK1* mutation, P55S, and AP. A trend did emerge for the N34S mutation to be more common in patients with severe AP (9.1%). This, however, failed to reach statistical significance, and further study is warranted before considering the N34S mutation as a risk factor for severe AP. In summary, in this study, as many as 7.8% of patients with AP were carriers of the *SPINK1* N34S mutation. This suggests that the *SPINK1* gene plays a role in susceptibility of AP.

Gene polymorphisms

Contrary to previous findings, we can demonstrate no differences between our study groups of mild and severe AP and controls. Furthermore, the group of patients with OF and similarly the group of severe-AP patients with infectious complications did not show different genotypes from those of controls. Thus, determination of these polymorphisms may be of no value in predicting disease severity in AP.

Several case-control studies present associations of AP with *TNF* -308 G/A polymorphism. *TNF* -308 G/A polymorphism has been associated with early septic shock and with severe AP (Table 2). Our data on 214 AP patients with alcohol-induced disease adds a considerable amount of evidence that *TNF* -308 G/A polymorphism is not associated with risk for the disease nor with its severity in alcohol-induced cases. It can

thus be assumed that this negative finding between risk and severity is valid also for other etiologies such as biliary and idiopathic AP, contrary to some earlier findings.

Our results are not in accordance with the association of *HSPA1B* +1267 polymorphism with severity of AP as presented by Balog and colleagues. This is not surprising, because conflict among sequential studies is a common phenomenon in case-control studies.^{261, 262, 263} Classification of severe AP followed different rules in the present study (Atlanta classification) and in the study of Balog and colleagues. (Ranson classification). Locus heterogeneity offers one explanation; we, however, did a subgroup analysis of different etiologies and found no differences between groups.

The most likely explanation for the association sometimes demonstrated in AP in the genes *HSPA1B* and *TNF* -308 is that haplotypes neighboring these SNPs are causative for the phenotype. Both these genes are located in chromosome 6, near the MHC genes. Indeed, in alcoholic CP, association with *TNF* microsatellite haplotypes may occur.²⁶⁴ *HSPA1B* intronic +1267 polymorphism does not alter the structure of the protein, and may be a marker of haplotypes associated with multiple diseases.^{265, 266, 267, 268} The positive associations found in other diseases encourage us to go further with studying *HSPA1B* gene haplotypes.^{269, 270} Other haplotypes of the *TNF* gene may be important, as demonstrated in other diseases.^{271, 272, 273}

Now we have very strong evidence that *CD14* -159 C/T polymorphism is not associated with disease outcome in AP.^{20, 198, 200, 201} None of these studies suggests that *CD14* -159 C/T polymorphism is associated with AP or complications. Chao and colleagues report from Taiwan that the *CD14* -159 C allele is associated with alcoholic AP.²⁴ In our patient sample, we could not confirm this finding. The patient population in the Chao study consumed high amounts of alcohol: a reported 140 g/day for 15 years. The amount of alcohol consumed is also high in reports associating the T allele with liver cirrhosis. We were unable to record the amount of alcohol consumed by our patients, so we only assume that their alcohol consumption exceeded 80 g/day, and quite often AP is associated with a binge-drinking pattern. Thus, our results do not rule out the possibility that *CD14* -159 C/T polymorphism can play a role in organ damage in long-lasting exposure to alcohol.

Based on our results, the *FV* Leiden mutation does not affect AP severity. Carriers of the Leiden mutation were evenly distributed among those with mild AP, severe AP, and severe AP with infectious complications or OF. The allele frequency for the Leiden mutation in this study was 2% both in patients and in controls, which is very comparable with earlier reports from our country.^{274, 275}

Our finding is the underexpression of the *PAI-1* G4 allele among those with severe AP ($p < 0.05$). Furthermore, the median hospital stay was shorter among those with the 4G/4G genotype. This finding is very contrary to our hypothesis. The *PAI-1* 4G allele is associated with higher PAI-1 levels and favors coagulation. High PAI-1 levels in AP have been associated with mortality.²⁷⁶ The 4G/4G homozygosity has been associated with obesity, a risk factor for severe AP.²⁷⁷ All in, it can be concluded that the *PAI-1* 4G/5G insertion/deletion polymorphism is not associated with disease outcome in AP.

Adipokines

Our results suggest that adiponectin and leptin plasma concentrations on admission are not involved as determinants of disease severity in AP, because plasma concentrations of adiponectin and leptin were similar in the groups of patients with mild and with severe AP. In AP patients matched by age, gender, BMI, and etiology, the on-admission plasma levels of adiponectin and leptin did not correlate with disease severity, suggesting that they do not affect the course of AP.

Future perspectives

The results of the present study show that there are, indeed, genetic factors involved in AP. Further identification of genetic factors in a multifactorial disease such as AP is becoming possible with employment of modern techniques in genetics. This requires clearly defined patient populations in regards also to the acquired risk factors in AP and even larger AP patient sample sizes with multicenter collaboration in patient collection. Employment of new technologies in genotyping enables the investigator to identify vast numbers of genetic variants. Analysis of the data requires advanced analytical and statistical methods that are available.

Further clarification of the complex trait of severe AP may be possible in clinical studies. Risk factors identified thus far are advanced age, comorbidities, and obesity. Features of the metabolic syndrome serve as a tempting starting point for study to identify additional factors associated with severity of AP. Detailed knowledge of clinical factors associated with the severity of AP aids in interpretation of future genetic studies of AP.

3.5 Conclusions

Results of the present study suggest that:

1. The *SPINK1* N34S mutation is a predictive factor in AP. This mutation appeared in 7.8% of our AP patients compared with 2.6% of controls, indicating that it is a risk factor for AP. The *SPINK1* N55S mutation showed no differences between study groups.
2. Inflammatory gene polymorphisms in genes *CD14*, *TNF*, *IL-10*, and *HSPA1B* have no influence on AP, since the polymorphisms showed no associations with severe AP or alcohol-induced AP compared with controls.
3. *FV* Leiden and *PAI-1* 4G/4G are not risk factors in severe AP because OF or infectious complications in severe AP were not more common in patients carrying these genotypes.
4. Plasma adipokines have no influence on systemic inflammation in clinical AP, since adiponectin and leptin plasma levels on admission to hospital did not differ between patients with mild AP and severe AP.

The identification of genetic factors associated with severe AP requires large patient populations with precisely characterized clinical risk factors, as well as familiarity with the pathogenesis of the disease. AP represents a genetically complex disease with evidently a heterogenic genetic background.

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